(2)

Handbook of NATURAL TOXINS

Volume 4
BACTERIAL TOXINS

Edited by

M. CAROLYN HARDEGREE
Bureau of Biologics
U.S. Food and Drug Administration
Bethesda, Maryland

ANTHONY T. TU
Department of Biochemistry
Colorado State University
Fort Collins, Colorado

MARCEL DEKKER, INC. New York and Basel

4

Enterotoxins of Staphylococci

Leonard Spero, Anna Johnson-Winegar,* and James J. Schmidt†

U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland

*Current affiliation: U.S. Army Medical Material Development Activity, Fort Detrick, Frederick, Maryland.

†The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

I. INTRODUCTION

The staphylococcal enterotoxins have been known for many years to be a major cause of food poisoning. Typical symptoms include vomiting and diarrhea 4–6 hr after ingestion of contaminated food. In 1963, Casman, Bergdoll, and Robinson introduced a serological basis for differentiating the various enterotoxins: A (Casman, 1960), B (Bergdoll et al., 1959), and C (Bergdoll et al., 1965a) had been identified. Later, enterotoxins C_2 (Avena and Bergdoll, 1967), C_3 (Reiser et al., 1984) (variants of enterotoxin C), D (Casman et al., 1967), and E (Bergdoll et al., 1971) were identified. Although these proteins are antigenically distinct, they are classie enterotoxins, as evidenced by biological activity in test animals. [A report by Bergdoll et al. (1981) tentatively identified a protein produced by some strains of *Staphylococcus aureus* cultured from toxic shock patients as enterotoxin F. More recent work has, however, shown that the etiologic agent of menstrual toxic shock syndrome is not an enterotoxin (Reiser et al., 1983).]

An extensive review article in 1970 by Bergdoll covered the basic information of these staphylococcal enterotoxins, and in this chapter we propose to deal primarily with those areas in which significant new information has become available.

II. EPIDEMIOLOGY

A recent annual summary from the Centers for Disease Control (1981) states that 460 outbreaks (13,207 cases) of food-borne disease were reported in 1979. Of those with confirmed bacterial etiology, *S. aureus* was the second most common cause, accounting for 34 outbreaks and 2391 cases. Results from 1975 to 1978 indicated a similar trend; that is, *S. aureus* enterotoxins were positively identified in about 15–24% of all reported cases (only *Salmonella sp.* caused more illness). Foods most often implicated are those such as meat salads (turkey, ham, etc.) that contain mayonnaise, and some dairy-type products, such as custard-filled pastries. Although most often isolated from foods, enterotoxin-producing strains of *S. aureus* have also been found in patients with pseudomembranous enterocolitis, scalded-skin syndrome, and osteomyelitis. Frequently, contamination of food may be traced to a food handler with an infected finger who neglected routine hygienic precautions.

Since most laboratories rely on serological methods of detection, known enterotoxin types can be identified fairly easily. Still unidentified serotypes probably do exist, but a great deal of work is involved in purifying these new toxins and preparing specific antisera.

III. PRODUCTION AND ISOLATION

A. Production

Each of the serological types of staphylococcal enterotoxin has been isolated in an essentially homogeneous state. Not surprisingly in view of the great similarity in molecular size, chemical structure, and properties, the methods used for production and isolation of the several types follow basically

TABLE 1 The Variation of Yield of Enterotoxin with Serotypes and Strain

Type	Strain	Amount of enterotoxin (μg/ml)
A	S-100	3
A	13N-2909	100
B	S-6	100–200
B	10-275	>500
C_1	137	60
D	1151m	0.5–1.0
E	FRI 326	3–5

the same pattern (SEA,* Schantz et al., 1972; SEB, Schantz et al., 1965; SEC_1, Borja and Bergdoll, 1967; SEC_2, Avena and Bergdoll, 1967; SEC_3, Reiser et al., 1984; SED, Chang and Bergdoll, 1979; SEE, Borja et al., 1972). The ease of purification is related directly to the yield of enterotoxin in the bacterial culture.

1. Strain

A striking difference occurs in the amount of enterotoxin obtained both within strains producing a given type and among maximum producers in different types. This is illustrated in Table 1. The highest producing strain, 10-275 of SEB, elaborates more than 100 times the amount of enterotoxin than strain 1151m of SED. However, experience with types A and B indicates that yields can be significantly improved by appropriate selection and mutagenic techniques. Strain 10-275 was derived from the parent isolate S-6 by colony selection and exposure to ultraviolet radiation, which resulted in a 5-fold augmentation of SEB production. Mutagenesis with N-methyl-N-N'-nitro-N-nitrosoguanidine of strain S-100 by Friedman and Howard (1971) produced a 20-fold increase of SEA, and in our laboratory colony selection from the mutant 13N-2909 has resulted in an additional 2-fold improvement.

2. Media

The dual goal of simplicity of medium and maximum yield of enterotoxin has been achieved with strikingly similar formulations. In every instance, the major ingredient is a proteolytic digest of casein such as one of the N-Z Amines manufactured by Sheffield and/or Protein Hydrolysate Powder prepared by Meade Johnson. Some of the enterotoxins also require

*Abbreviations used in this chapter are as follows: SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC_1, SEC_2, and SEC_3, variants of staphylococcal enterotoxin C; SED, staphylococcal enterotoxin D; SEE, staphylococcal enterotoxin E.

dextrose for optimum yield. The only vitamins required are niacin and thiamine. The starting pH differs slightly for the several types but is always near neutral.

3. *Fermentation*

Since the initial reports of the production of enterotoxin A, B, and C, a significant improvement in fermentation techniques has occurred through the use of industrial fermenters with automated controls. Initially, the medium is autoclaved in the fermenter vessel and a tape record of the time and temperature is made. During the fermentation, parameters such as the rate of air sparging, stirring, and the addition of antifoam are controlled and monitored to provide a continuous recording of the pH, temperature, and oxygen tension. The precise control of cultural conditions with this instrumentation is in large measure responsible for the success in producing decagram amounts of enterotoxins A, B, C_1, and C_2.

4. *Concentration*

The first and one of the most difficult problems in isolation of the enterotoxin is the reduction of the very large volumes involved in the fermentation to volumes suitable for laboratory manipulation. At the Food Research Institute of the University of Wisconsin, dialysis against Carbowax or polyethylene glycol has been extensively employed. In our laboratories, the method of choice has been a bulk adsorption onto an ion-exchange resin followed by transfer of the resin to a chromatographic column and then elution by a strong buffer.

B. Purification

Ion-exchange chromatography and gel filtration have been the principal techniques used for purification of these toxins. A description of the precise methodologies for seven different toxins is not possible in this chapter; we have, however, recently reported in detail the procedure used in our laboratories for the production and isolation of SEA (Spero and Metzger, 1981). These methods are a modification of the publication of Schantz et al. (1972) and represent our experience over a period of more than 10 years. This summary is presented to give a general idea of the purification procedures for the entire group of enterotoxins.

1. A single lyophilized ampule of the high-producing strain 13N-2909 of *S. aureus* is used for each fermentation. The culture is passed three times in shake flasks at 37°C for 18–24 hr to obtain 200 ml of inoculum.
2. A 70-liter fermenter (Fermentation Design, Inc.) containing 50 liters of sterile medium consisting of 4% N–Z Amine type NAK (Sheffield), 1% yeast extract (Difco), and 0.2% dextrose is inoculated. Fermentation conditions are agitation at 400 rpm, sparging at a rate of 10 liters/min of air, and the addition on demand of antifoam (a 50% suspension of Antifoam 60, Harwick) at 37°C for 18–24 hr.
3. The bacterial cells are removed by centrifugation at 20,000 rpm in a continuous-flow system (Lourdes), and the supernatant is diluted fourfold with water.

TABLE 2 Summary of the Purification of Staphylococcal Enterotoxin A

Purification steps	Volume (ml)	Toxin (mg/ml)	Recovery (%)	Purity (%)
Centrifuged culture	50,000	0.09–0.10	—	—
CG-50	3,000	0.25–0.35	15–20	45–60
CM-Cellulose	300–450	0.90–1.4	50–70	80–90
Hydroxyapatite	500–600	0.75–1.2	90–95	>99

4. About 1 lb of the strong carboxylic acid exchanger, CG-50 (Rohm and Haas), is stirred into the clear supernatant and after 2 hr is washed and transferred into a chromatographic column. The column is eluted with 0.5 M phosphate buffer at pH 6.2 containing 0.5 M NaCl. The toxin is contained in the second and major peak.
5. After dialysis to reduce the salt content, the toxin is adsorbed in bulk onto 200 g of carboxymethylcellulose. The cellulose is transferred to a chromatographic column and eluted with a linear gradient of phosphate buffer from 0.01 M at pH 6.0 to 0.05 M at pH 6.8. The toxin-containing peak is clarified by centrifugation.
6. The toxin solution is applied to a column of hydroxyapatite and eluted with a linear gradient from 0.2 to 0.4 M phosphate at pH 5.7. The process is summarized in Table 2 (Spero and Metzger, 1981). From the original volume of 50 liters containing some 5 g of enterotoxin, an overall yield of about 10% or 0.5 g of highly purified material is obtained.

C. Purity

As judged by most physicochemical criteria, the isolated enterotoxins are essentially pure proteins. Only a single component is observed by analytical ultracentrifugation, ion-exchange chromatography, gel filtration, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In addition, the final products possess high specific activity, indicating that little or no inactivation has taken place during the purification process. SED is a possible exception to this. Although it is clear that the isolated component is indeed the enterotoxin, a precise specific biological activity is not available. In the opinion of Chang and Bergdoll (1979), SED "would appear only slightly less toxic than the others."

A significant heterogeneity is apparent in purified SEB in starch gel electrophoresis (Baird-Parker and Joseph, 1964). This was confirmed by Schantz et al. (1965), and later four species were demonstrated by the use of isoelectric focusing in carrier ampholytes stabilized by a sucrose gradient (Metzger et al., 1972). A better separation is obtained by isoelectric focusing in polyacrylamide gels as shown in Fig. 1. A similar heterogeneity is also seen in SEA, SEC_1, SEC_2, and SEC_3 and is likely to be present in SED and SEE, although this has not yet been substantiated. Chang and Dickie (1971) reported that the component of SEB present in highest concentration is partially converted after isolation to the next more

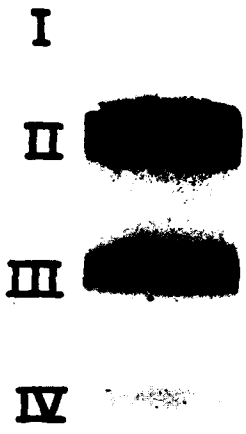

FIGURE 1 Microheterogeneity of staphylococcal enterotoxin B demonstrated by isoelectric focusing in polyacrylamide gel. The components are numbered arbitrarily in order of decreasing isoionic points.

acidic species and attributed the alteration to amide hydrolysis. The validity of the hypothesis was shown conclusively by Spero et al. (1974). A comparison of the measured isoionic points of the four components of SEB with values calculated for varying numbers of excess basic groups indicates that each component differs from the succeeding one by a single charge. Sequential conversion of the components from the more to the less alkaline forms is obtained by exposure to pH 9.0 and 37°C for 8 days. This interconversion is shown in Fig. 2. In this experiment, the enterotoxin used was a fraction isolated by isoelectric focusing containing virtually only the two more prominent components II and III. Component II decreased steadily, component III increased slightly, and component IV increased greatly. There was also the appearance of a new, more acidic entity, component V. The data give an excellent fit for two consecutive first-order reactions in which the specific reaction rate constants are nearly identical. Finally, it was demonstrated chemically that ammonia is liberated under the conditions used for the conversion. Although amide hydrolysis is clearly the mechanism for these conversions, the rate is too slow to account for the appearance of the four components during the comparatively short period of bacterial fermentation. It was suggested that only the most cathodic component is synthesized by the organism and that this is converted to the other forms enzymatically.

This microheterogeneity was investigated by Metzger et al. (1975) for SEC_1 and SEC_2. SEC_1 is more markedly affected by exposure to alkaline conditions than is SEC_2. A bacterial deamidase was again postulated to act during the fermentation period.

FIGURE 2 Conversion of more to less alkaline forms of staphylococcal enterotoxin B by exposure to pH 9.0 and 37°C. The samples were isoelectrically focused on polyacrylamide gels with pH 3–10 ampholines. The gels are from left to right for 0, 1, 2, 4, and 8 days. The components are numbered as in Fig. 1 (Spero et al., 1974).

IV. STRUCTURE AND FUNCTION

A. Basic Structure

All of the staphylococcal enterotoxins have the same basic structure—a single polypeptide chain containing a single disulfide loop. They contain no carbohydrate, lipid, or nucleotides. The molecular weights are within the range of 27,500–30,000. Their isoelectric points vary from just below neutral to pH 8.6 and are consistent with the number of basic and acidic amino acids.

B. Primary Structure and Active Site

The native enterotoxins B and C_1 are highly susceptible to limited proteolytic cleavage by trypsin, with full retention of immunological and emetic activities (Spero et al., 1973, 1976). This property was exploited to investigate structure-function relationships within the molecules and is discussed in detail below. The high proteolytic labilities of certain peptide bonds in types B and C_1 suggested a mechanism of activation similar to diphtheria or botulinum toxins, but the observation that native enterotoxin A is completely resistant to trypsin-catalyzed hydrolysis (Spero et al., 1973) does not support this hypothesis.

Incubation of native enterotoxin B with trypsin at pH 9.0 results in the cleavage of a single peptide bond between lysine-98* and threonine-99 (Fig. 3).

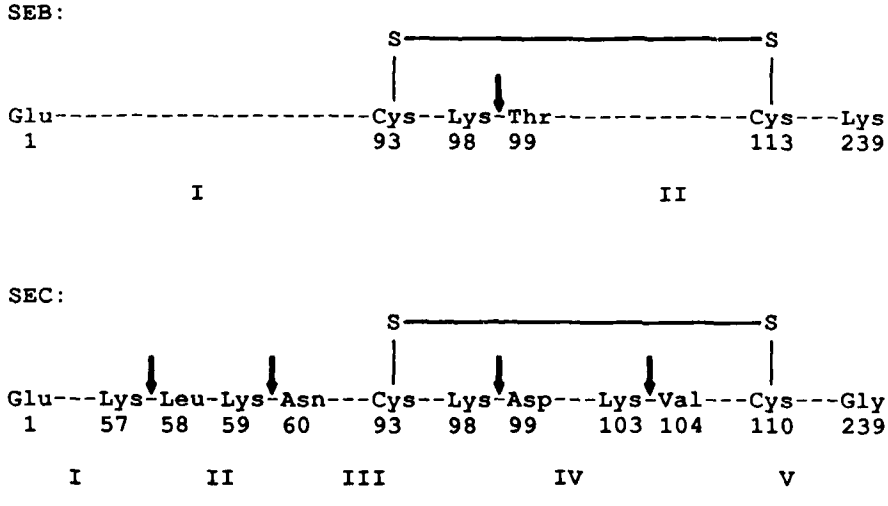

FIGURE 3 Schematic representation of the peptides produced by the action of trypsin on native staphylococcal enterotoxins B and C_1. The cleavage sites are indicated by arrows (Spero et al., 1973; Schmidt and Spero, 1983).

*The sequence numbering of SEB used herein is based, except where otherwise indicated, on the sequence deduced from its DNA sequence by Jones and Kahn (1986).

This site is located within the 21-residue disulfide loop, and the nicked toxin exhibits the physical properties of a single particle with no change in toxicity. Subsequently, the same results were found after reduction and carboxamidomethylation of the disulfide bond; that is, the two polypeptides of nicked toxin remain associated and biologically active even though the disulfide loop is cleaved and alkylated (Spero et al., 1973, 1975b).

Separation of the two constituent polypeptides of nicked enterotoxin B can be accomplished by chromatography of the reduced and alkylated derivative on CM-cellulose in 8.0 M urea (Spero et al., 1975b). Upon removal of denaturant, the individual peptide chains are not emetic. However, the two chains can reassociate to form a molecule that is essentially identical to the parent nicked toxin with respect to molecular weight, mitogenicity, serologic and emetic activity.

In contrast to type B, incubation of native enterotoxin C_1 with trypsin results in the proteolysis of four peptide bonds (Spero et al., 1976; Schmidt and Spero, 1983). The cleavage pattern is depicted schematically in Fig. 3. The nicked derivative exhibits emetic, mitogenic, serologic, and physical properties that are essentially identical to those of the parent molecule, and the three largest constituent peptides (I, III, and V in Fig. 3) do not dissociate in the absence of denaturant. Using gel chromatography in 6.0 M guanidine, Spero and Morlock (1978) purified peptide I and also a larger fragment consisting of peptides III and V joined via the disulfide bond. The latter molecule is emetic but not mitogenic. The converse is true for peptide I.

In the same study, peptides III and V were individually purified after reduction and alkylation of the disulfide bond. However, peptides II and IV were overlooked. In a modification of the purification, 10% (v/v) acetic acid was substituted for 6.0 M guanidine, and this led to the recovery of all five peptides (Schmidt and Spero, 1983).

Chemically determined complete covalent structures are available only for types B (Huang and Bergdoll, 1970) and C_1 (Schmidt and Spero, 1983). The sequence of type B has now also been deduced from the sequence of its DNA (Ranelli et al., 1985; Jones and Kahn, 1986). While several discrepancies were found, most of them were due to erroneous assignments of amide and free carboxy groups in the earlier work. The sequences of the two types are compared in Fig. 4. Considerable homology is seen, particularly in the C-terminal region, where 59 of the last 67 residues are identical. However, arranging the two sequences in a strictly parallel manner did not always result in maximum homology. A gap of three residues was required in the disulfide loop of SEC_1 to make the second half-cystines coincide. Additionally, two gaps were needed in other areas of the type B chain.

The scope and accuracy of structure-function relationships within the staphylococcal enterotoxins would be enhanced by comparing the amino acid sequences of types B and C_1 with that of another serotype with more extensive differences in primary structure. Biophysical and immunological data suggest that enterotoxin A is an excellent candidate in this regard, and its sequence has recently been derived from its DNA sequence (M. Betley, personal communication). At this writing, however, it has not been published, and only limited portions of a chemically derived sequence (Huang et al., 1975) are presently available. These are compared with corresponding areas of types B and C_1 in Fig. 5. Again, regions of homology are seen, and it has been proposed (Huang et al., 1975) that

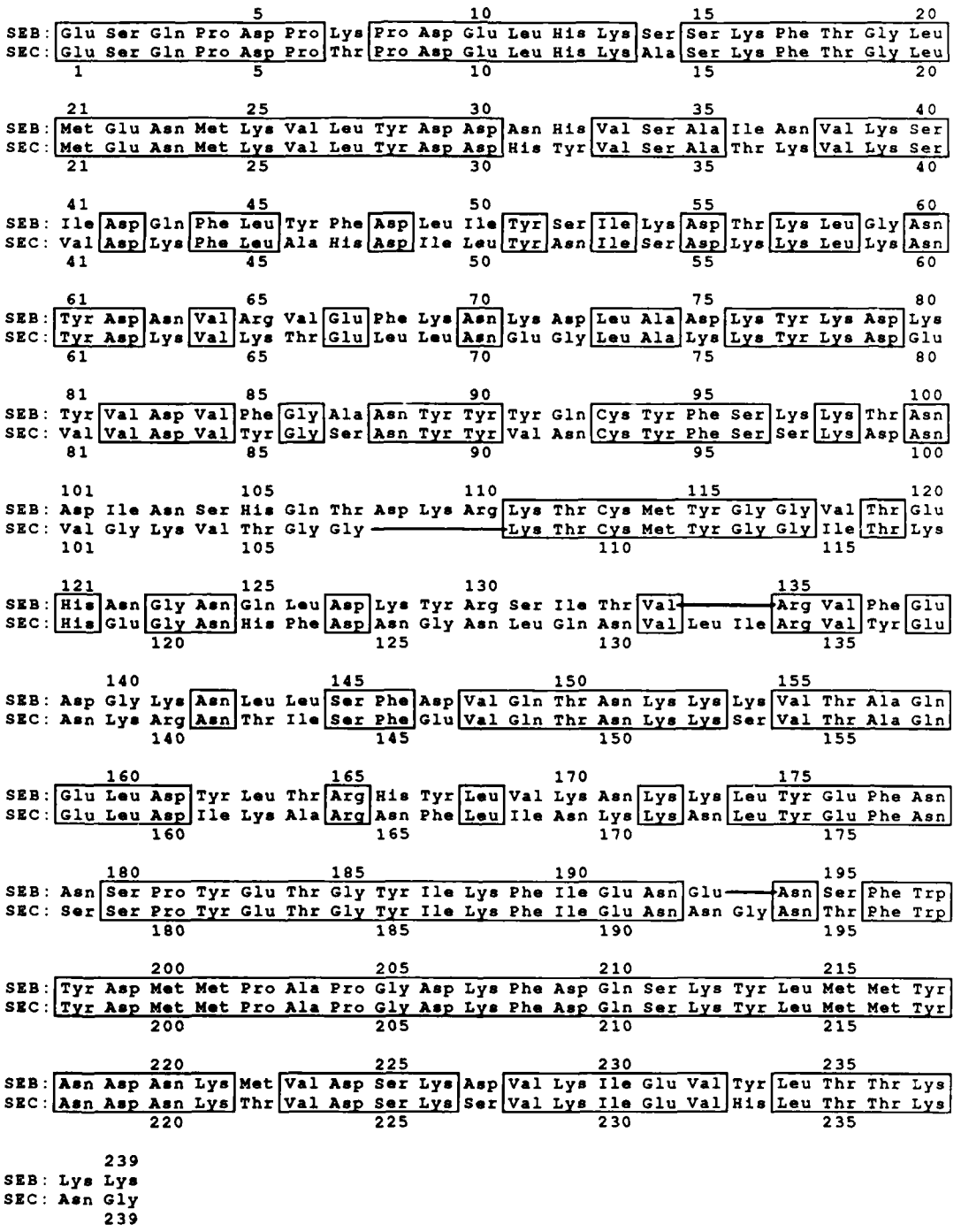

FIGURE 4 A comparison of the amino acid sequences of SEB (Jones and Kahn, 1986) and SEC_1 (Schmidt and Spero, 1983). Boxes are used to highlight identical residues.

```
SEA: Val Asp Leu Gly Ala Tyr Tyr Gly Tyr --- Gln Cys
SEB: Val Asp Val Phe Gly Ala Asn Tyr Tyr Tyr Gln Cys
SEC: Val Asp Val Tyr Gly Ser Asn Tyr Tyr Val Asn Cys
                  85                    90

SEA: Thr Ala Cys Met Tyr Gly Gly Val Thr Leu His Asp Asn Asn Arg Leu Thr Glu Glu Lys
SEB: Lys Thr Cys Met Tyr Gly Gly Val Thr Glu His Asn Gly Asn Gln Leu Asp Lys Tyr Arg
SEC: Lys Thr Cys Met Tyr Gly Gly Ile Thr Lys His Glu Gly Asn His Phe Asp Asn Gly Asn
            110                 115                 120                 125

SEA: Arg Asp Asn Lys Thr Ile Asp Ser Glu Asn Met His Ile Asp Ile Tyr Leu Tyr Thr Ser
SEB: Asn Asp Asn Lys Met Val Asp Ser Lys Asp Val Lys Ile Glu Val Tyr Leu Thr Thr Lys Lys Lys
SEC: Asn Asp Asn Lys Thr Val Asp Ser Lys Ser Val Lys Ile Glu Val His Leu Thr Thr Lys Asn Gly
            220                 225                 230                 235
```

FIGURE 5 A comparison of the partial sequence of staphylococcal enterotoxin A (Huang et al., 1975) with corresponding areas of SEB and SEC_1. Homologous residues are in boldface. Residue numbers are those of SEC_1.

the 14-amino acid segment of the sequence beginning with the second half-cystine composes the active site of the toxins.

Homology with the C-terminal region of SEC_1 and SEB has now also been found in the sequence of the type A pyrogenic exotoxin from *Streptococcus pyogenes* (Weeks and Ferretti, 1986; Johnson et al., 1986). Furthermore, a sequence segment following the last half-cystine is highly conserved. Whether these similarities have biological significance is uncertain, but it is noteworthy that the molecules share many properties such as lymphocyte mitogenicity, pyrogenicity, and enhancement of susceptibility to endotoxin shock.

C. Modification Studies

A clarification should be introduced before a discussion of these studies. It is not intended that "active site" be interpreted in its traditional sense. Rather, in view of our lack of knowledge of a precise mechanism of action, it is used here to connote any well-defined surface structure involved in a biological effect of the enterotoxins.

The disulfide loop is common to all the staphylococcal enterotoxins, but the disulfide bond itself cannot be part of an active site. SEB can be reduced and alkylated with no loss in toxicity (Spero et al., 1975b; Dalidowicz et al., 1966). Moreover, the immediate environment of the two half-cystines is dissimilar in types A, B, and C_1. Enterotoxin C_1 is resistant to reduction by mercaptoethanol under conditions where the other two are completely reduced (Spero, 1981) and the circular dichroism of the loops of SEB and SEA are quite different. Additionally, the amino acids comprising the loop are unlikely to be involved in the active site, since in type C_1 this segment is three residues shorter than in the B variant. Further, it was found that the trypsin-treated derivative of type C_1 retains full biological activity (Spero et al., 1976), in spite of the fact that this molecule lacks residues 99–103 from within the loop.

Studies employing chemical modification of specific residues have provided very little definitive evidence on the nature of the active site. For example, it has been shown that six and five chemically available and normally titrating tyrosines of SEB and SEC_1, respectively (Chu, 1968; Berjak, 1969), may be acylated without affecting either combining power with antibody or emetic activity. While one may guardedly infer that these particular residues are not part of antigenic determinants or the active site, there is at present no information identifying which of the large number of tyrosines in the amino acid sequences are involved. Similar considerations apply to the observations of Chu and Crary (1969), that 21 to 24 of the 33 carboxyl groups of SEB could be blocked by reaction with glycine methyl ester with little or no effect on the emetic activity.

In the study of the oxidation of the methionine residues by hydrogen peroxide, Chu and Bergdoll (1969) demonstrated that when six residues of SEB were modified, the emetic activity was lost. However, the discovery that only one methionine residue is conserved in SEA, SEB, and SEC (Fig. 5, residue 111) clearly limits any deductions from those data.

When the free amino groups of SEB were acetylated or succinylated, the combining power and the emetic activity were reduced proportionally to the number of groups modified (Chu et al., 1969). However, up to 90% of the lysine residues could be guanidinylated with O methyl isourea without loss of either activity. It was concluded that the positive charges

contributed by the amino groups in SEB play an important role in the antigen-antibody reaction and in the emetic activity. Spero et al. (1971) used a more effective reagent to guanidinylate SEB and achieved a product with 31–32 of the 33 free amino groups modified with retained full serologic and emetic activity. Their conclusion, however, was that the great increase in group size that occurs in converting a lysine to a homoarginine (see Wofsy and Singer, 1963) would serve to eliminate those groups as part of a combining site in the antigen-antibody reaction. This interpretation was extended also to participation in an active site for emesis, but it was recognized that extremely little is known about the active site or about the nature of the receptor and that much more flexibility in an enterotoxin-receptor complex may be permissible than in the union of an antigen and an antibody.

The two tryptophan residues in SEA are readily oxidized by N-bromosuccinimide, but the single tryptophan residues of types B and C_1 are resistant (Spero, 1981). It was concluded that tryptophan could not be part of an active site and the location of the tryptophan residues of SEA in positions far removed from that in SEB and SEC_1 (I.-Y. Huang, personal communication) provides an unequivocal confirmation.

Stelma and Bergdoll (1982) reported that alkylation of five or six histidine residues in SEA leads to a loss of emetic activity with little change in molecular conformation. Scheuber et al. (1985) extended this observation to SEB. They were able to completely carboxymethylate its six histidine residues and to demonstrate a loss of toxicity without affecting the immunological specificity. Examination of the complete amino acid sequences of SEB and SEC_1 and the available information on SEA reveals that only one histidine residue is conserved in all three enterotoxins and that this residue is in the highly conserved segment following the second half-cystine proposed as the active site of the toxins (Huang et al., 1975). Moreover, the secondary structure of SEB (see Fig. 8, below) places this histidine in a highly predicted β-turn and therefore likely to be on the surface of the molecule (Kuntz, 1972).

It should also be noted that the pyrogenic exotoxin of *S. pyogenes* has a histidine residue in a comparable sequence segment (Weeks and Ferretti, 1986; Johnson et al., 1986).

D. Conformation

Physical parameters of SEB such as sedimentation coefficient, diffusion coefficient, and intrinsic viscosity indicate that the molecule is a prolate ellipsoid with an axial ratio of approximately 2:1 (Wagman et al., 1965; Bergdoll et al., 1965b). Although complete physical data are not available for the other serotypes, what has been obtained is consistent with this model.

Efforts to probe the secondary structures of the enterotoxins have been made through the use of circular dichroism (CD). Muñoz et al. (1976) examined SEB and Middlebrook et al. (1980) compared the spectra of SEA, SEB, and SEC_1. The latter are presented in Fig. 6 for the near ultraviolet and in Fig. 7 for the far ultraviolet. The CD spectra of SEB and SEC_1 are almost identical from 250 to 320 nm. Superficially, the spectrum of SEA does not appear to have much in common with them, but a close examination reveals that most of the bands are conserved with respect to both location and sign. Spero (1981) was subsequently able to

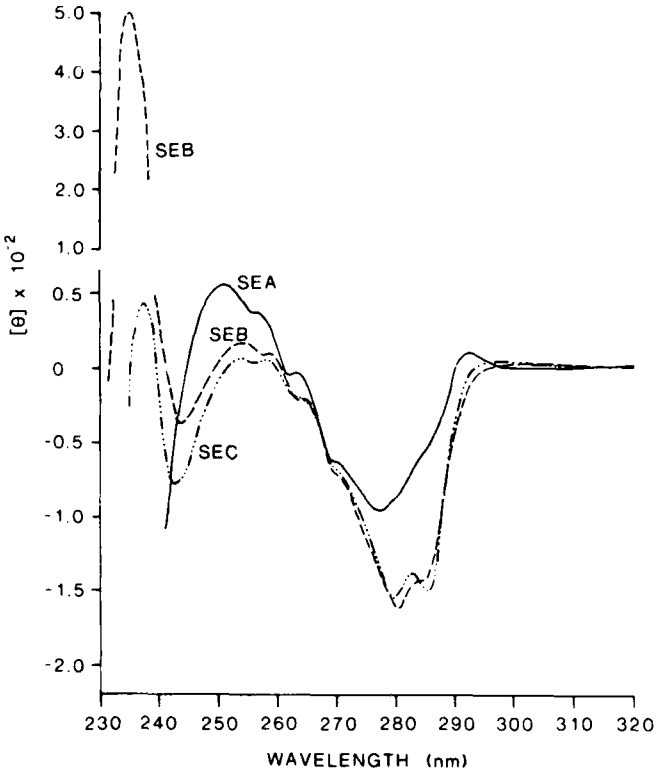

FIGURE 6 A comparison of the aromatic region circular dichroism of staphylococcal enterotoxins A, B, and C_1 (Middlebrook et al., 1980).

resolve the spectra in this region empirically into Gaussian curves. Each spectrum contained the same six components with maximum ellipticities at virtually identical wavelengths. The strength of the bands of SEA, however, differed significantly from those of SEB and SEC_1. Each Gaussian curve was identified as corresponding to a prominent CD transition of an aromatic chromophore.

The CD spectra of SEB and SEC_1 are also much more alike from 190 to 250 nm. Although all three proteins have a major negative extremum at 215–218 nm, its magnitude is equal in enterotoxins B and C_1 but substantially decreased in enterotoxin A. SEA also lacks the positive band at 235 nm so prominent in SEB and SEC_1. These differences in the peptide-absorbing region are indicative of differences in the manner of folding of the elements of the secondary structure of SEA; nevertheless, the analysis described above is surely suggestive of a similar environment of the aromatic residues in the tertiary structure. It seems likely that the basic folding of the enterotoxins is the same, with at least an identity of folding of that part of the molecule required to generate the biologically active site.

Attempts to analyze the secondary structures of the far ultraviolet CD spectra in terms of α-helix, β-forms, and aperiodic conformation content gave unsatisfactory computer fits. One consistent result was a low α-helix

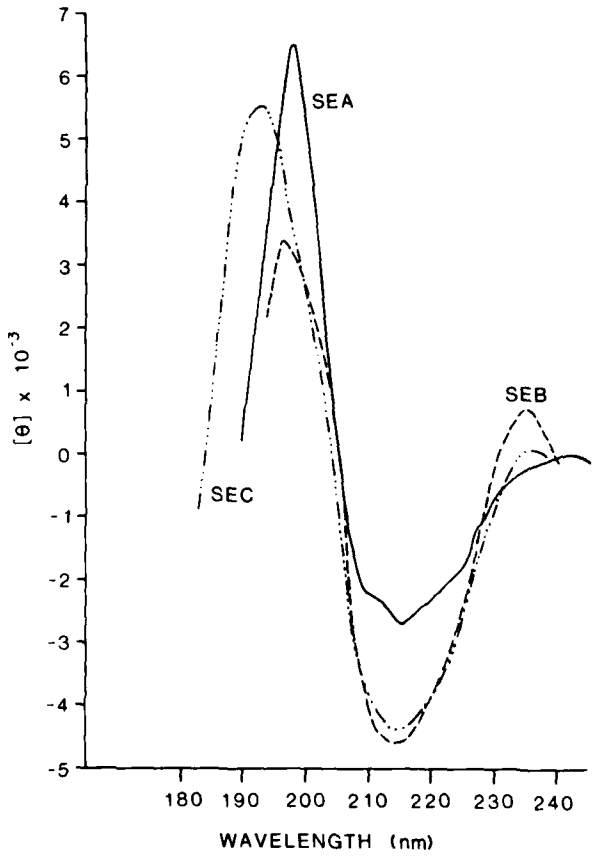

FIGURE 7 A comparison of the peptide region circular dichroism of staphylococcal enterotoxins A, B, and C_1 (Middlebrook et al., 1980).

content (less than or equal to 10%) for all three enterotoxins. Otherwise, aperiodic conformation seemed to dominate. (Muñoz et al., 1976, were able to obtain a fit using a more limited part of this spectrum and estimated 11% α-helix, 35% β-sheet, and 55% random in SEB.)

Predictions of the secondary structure of SEB have been made based on the known primary sequence of the protein (Muñoz et al., 1976; Middlebrook et al., 1980). A linear representation from the latter authors is shown in Fig. 8. It places 29 residues in α-helix, 71 in β-sheet, 88 in β-turns, and 55 in aperiodic conformation. (Four residues are indicated in two structural elements.) The percentage composition is 12% α-helix, 30% β-pleated sheet, and 58% in β-turns or aperiodic conformation. These values are consistent with those calculated from the experimental CD data.

The most striking feature of the structure is the extensive region of β-sheet around the disulfide bridge. Richardson (1981) has observed that over half of all disulfide bridges in known tertiary protein structures are associated with a peptide backbone in extended β-conformation. The large number of predicted β-strands might suggest a β-barrel tertiary architecture. Warren et al. (1974), based on a study of the stability of

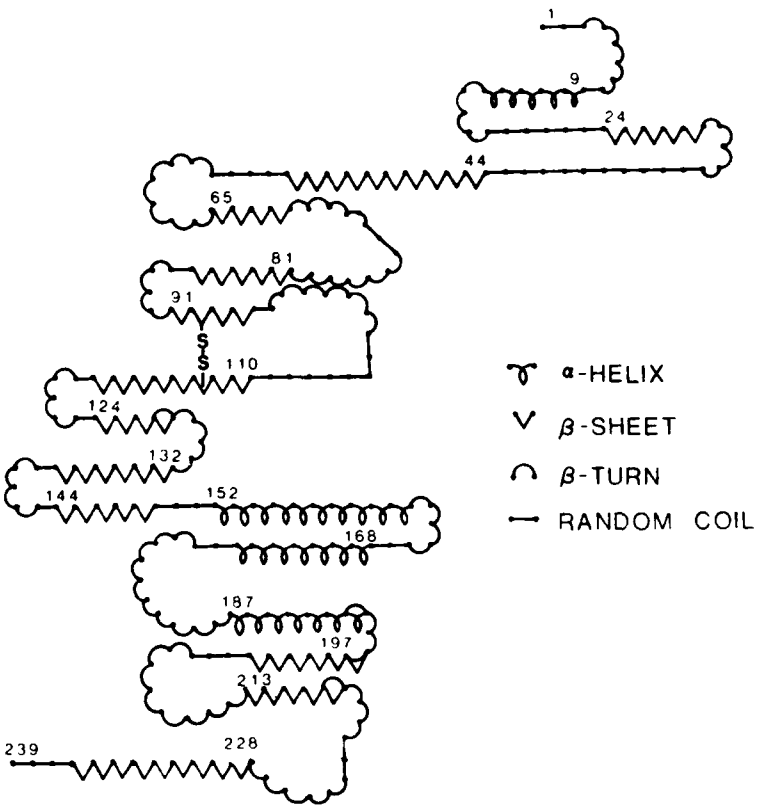

FIGURE 8 Schematic diagram of the secondary structure predicted for SEB. Each symbol represents one residue (Middlebrook et al., 1980). Residues are numbered from the sequence of Huang and Bergdoll (1970).

disulfide bond of SEB, proposed that there are two domains in the molecule, one being the disulfide loop.

These speculations are all that can presently be said about the tertiary folding of the enterotoxins. Recently, however, crystals of SEB suitable for x-ray diffraction have been obtained (M. Sax, personal communication).

V. DETECTION METHODS

Data based only on confirmed cases demonstrate that staphylococcal intoxication represents one-fourth of all food-borne disease outbreaks in the United States. Enterotoxin A is most often associated with illness in humans, but all the known serotypes have been implicated (Centers for Disease Control, 1981). The minimal dose of toxin required to produce illness is estimated to be <1 μg/kg via the oral route of ingestion.

The development of rapid, sensitive methods to survey samples (food especially) for the presence of the preformed bacterial enterotoxins is of considerable public health importance and has been the object of research

in many laboratories. Serological assays depend ultimately on the quality of the antisera and the purity of the toxins used to prepare them. However, even when the antigens are homogeneous, an intrinsic cross-reactivity between several serotypes causes some problems in identification (see Sec. VIII.A). Interference from other materials in food samples has proved to be another problem in assay development. Extraction procedures have often been employed, but with only limited success (Niskanen and Lindroth, 1976).

A comprehensive summary of detection methods was provided in an earlier review by Bergdoll (1970). Since then, a number of methods have been developed using radioimmunoassay (RIA) and enzyme-linked immunoadsorbent assay (ELISA) techniques. Both offer much higher sensitivity than previously published procedures.

The early RIA procedure developed by Johnson and co-workers (1971) bound the antibody to polystyrene tubes. These coated tubes presumably provided the advantage of indefinite storage at 4°C; however, the reproducibility between replicate tubes was not good (Berdal et al., 1982). Some investigators also felt that overnight incubation of the antigen-antibody complex was required in order to achieve reliable consistency in the test, precluding a rapid analysis.

The use of insoluble adsorbents, such as bromacetyl-cellulose (Collins et al., 1972) or the use of a double-antibody technique with antirabbit gamma globulin as co-precipitant did provide some improvement in reproducibility over the tube method. Here the major difficulties were the variability of the coupled antibody preparations and a decreased level of sensitivity. The use of protein A (in the cell walls of *S. aureus* Cowan I strain) as an immunoadsorbent (Jonsson and Kronval, 1974) provided a superior alternative to the above methodologies. The binding of protein A to the Fc region of the IgG is rapid, the separation of the cell-antibody complex is easily accomplished, and the preparation of the very stable protein A cells is inexpensive.

The main problem with all RIA procedures remains the availability of highly purified toxin and the radiolabeling of the toxin. Investigators have labeled the enterotoxins with ^{125}I or ^{131}I by means of lactoperoxidase (Robern et al., 1975), chloramine-T (Greenwood and Hunter, 1963), or gaseous diffusion (Gruber and Wright, 1967). In all methods, the labeled enterotoxin may be partially denatured, in addition to having a limited shelf life. Furthermore, RIA methods require use of rather expensive isotope-counting equipment and require special handling of radioactive materials.

The ELISA technique was first used for enterotoxin detection by Saunders and Clinard (1976) and Saunders and Bartlett (1977) with SEA. Simon and Terplan (1977) described a competitive system for SEB. Since then, many laboratories have explored the method, evaluating such parameters as coupling reagents, activation enzymes, chromogens, and monoclonal versus polyclonal antibodies. Fey, Pfister, and Ruegg (1984) compared four versions of the ELISA method for their suitability in detecting SEA, SEB, SEC, and SED. They found a sandwich technique with labeled antibody to be the best. Polystyrene spheres were coated with antibody and reacted with a sample containing toxin. The binding was detected with a second antibody coupled to phosphatase using *p*-nitrophenyl phosphate as substrate. The sensitivity was 0.1 ng/ml, and the test could be made quantitative. Interference by protein A was eliminated by the addition of normal rabbit serum. A commercial diagnostic kit is available.

Biological assay remains the only method for detecting new serotypes of enterotoxins. The most reliable bioassay is feeding suspected samples to young rhesus monkeys, as described by Bergdoll (1970). Intraperitoneal or intravenous injections of cats or kittens have been used to some extent, but cats are subject to nonspecific reactions (to other staphylococcal products). Many other species of animals have been tested, but none are as sensitive and reliable as the monkey. The criterion, then, for a new enterotoxin serotype is that administration of the purified protein will produce emesis and diarrhea in monkeys.

VI. SYNTHESIS

A. Cloning of Enterotoxin Genes

The gene of enterotoxin B was cloned in *Escherichia coli* using a vector plasmid (Ranelli et al., 1985). Initial insertions did not yield detectable SEB, but when the gene was placed downstream from a strong λ-phage promoter, the toxin was synthesized at readily detectable levels. The gene was also introduced into an enterotoxin-negative strain of *S. aureus*, and the clone was shown to produce SEB at levels comparable to the reference strain S-6. The gene has been located to a 2.1 kilobase-pair region.

Expression of the gene requires the *agr* gene product (S. A. Kahn, personal communication). When the cloned B gene was introduced into an agr^- strain, the resulting clones produced only about 1–5% of SEB levels produced by standard *S. aureus* strains containing the B gene. However, the levels of enterotoxin B mRNA in the agr^- strains were comparable to that present in the agr^+ strains, indicating that the *agr* gene regulates the expression of the B gene at a post-transcriptional or translational level.

Betley et al. (1984) have cloned the gene of enterotoxin A in *E. coli* using a vector plasmid. Antigenically active SEA was demonstrated from two clones. The toxin was extracted into the periplasmic space in the organism and could be released by osmotic shock. The gene is located within a 2.5 kilobase-pair fragment that is part of a discrete genetic element 8–12 kilobase pairs in length.

B. Membrane-Bound Toxin Precursor

Blobel and Dobberstein (1975) proposed a model system for protein secretion involving an additional sequence of amino acids usually located at the amino terminus of the protein. The putative purpose of this signal peptide is to facilitate passage across the cell membrane. In line with this work, Tweten and Iandolo (1981) reported the isolation from *S. aureus* membranes of a precursor form of enterotoxin B. In a continuing study (Tweten and Iandolo, 1983) using pulse-chase techniques, they were able to demonstrate both the precursor and the mature form of SEB after 5 sec. They concluded that post-translational and co-translational modification of the precursor were necessary to account for the rapid appearance of mature SEB. All of the precursor was associated with the membrane fraction. They also speculate that the mature SEB molecule may be sequestered somewhere in a cell wall compartment (protected from proteases) before final secretion.

A recent study from the same laboratory (Christianson et al., 1985) demonstrated a completely analogous situation with enterotoxin A synthesis, that is, a larger molecular weight precursor, association with the membrane, processing to a mature form, and transient association of the mature form with the cell wall.

Jones and Kahn (1986) have sequenced the precursor of SEB and identified a 27-amino acid-long signal peptide. The precursor has a molecular weight of 31,400, compared with the mature extracellular protein, which has a molecular weight of 28,366.

C. Plasmid Involvement in Enterotoxin B Production

In 1969, Dornbusch et al. demonstrated an association between methicillin resistance (Mec^R) and SEB production in a strain of *S. aureus* and suggested physical linkage of the responsible genes on a plasmid. Later, Shalita et al. (1977) confirmed that a small plasmid, since designated pSN2 with a molecular weight of 1.15×10^6, was essential for toxin production in the Dornbusch strain. However, Shafer and Iandolo (1978a) demonstrated that about 71% of *S. aureus* strains producing enterotoxin B do not contain this small plasmid. Genetic analysis indicated that in these strains, the gene for SEB production is chromosomal. Moreover, in further experiments using transformation and transduction with plasmid DNA, they could not demonstrate any physical linkage between Mec^R and SEB production. It appeared, therefore, that while in some Mec^R strains the production of SEB is associated with the presence of the 1.15 Mdal plasmid, in most strains the gene for SEB is definitely chromosomal.

Dyer and Iandolo (1981) then conducted a series of experiments to determine whether pSN2 contained the chromosomal structural gene. Using *Bacillus subtilis* minicells in a coupled translational assay system, they demonstrated that when pSN2 was introduced into *Bacillus subtilis*, a peptide of about 18,000 daltons was produced (indicating that pSN2 was being expressed in the system), but no transformants produced SEB. Their evidence, while highly suggestive, does not prove conclusively that pSN2 does not code for SEB production.

Unequivocal evidence has been provided by Kahn and Novick (1982). They have determined the entire nucleotide sequence (1288 base pairs) of pSN2 DNA. A coding sequence was identified for a polypeptide of 158 amino acids corresponding to a 20,000-dalton protein species expressed in minicells and in an in vitro protein synthesis system. (Presumably, this is the same protein reported by Dyer and Iandolo, 1981, to have a molecular weight of 18,000.) The amino acid sequence deduced from the DNA sequence does not resemble the amino acid sequence of SEB.

The question, then, is whether pSN2 plays a role in the control of SEB synthesis. Iandolo's group has contended that it does. They transformed a spontaneous SEB-negative derivative of a chromosomal SEB producer with pSN2 DNA and obtained SEB-positive cells (Dyer and Iandolo, 1981). However, these positive derivatives did not contain an autonomous pSN2 element. They contend that this is the result of an insertion of pSN2 at a chromosomal site. As supporting evidence, they present data that demonstrate pSN2 homology both in natural SEB producers and in genetically constructed chromosomal SEB producers (Dyer and Iandolo, 1982).

Khan and Novik (1982) conclude that it does not. They cured the Dornbusch strain of pSN2 by methods designed to avoid toxic chemicals or

other damaging agents and obtained derivatives that were SEB-positive. They then carried out DNA–DNA hybridization experiments to test the possibility that the pSN2 plasmid was integrated into the chromosome. All were negative. Finally, to explain the early results of Dornbusch et al. (1969), and Shalita et al. (1977), they suggest that the SEB determinant is a highly mobile genetic element, which they have named a hitchhiking transposon (Novick et al., 1980).

D. Genetic Analysis of Enterotoxin A Production

In 1978, Shafer and Iandolo (1978b) demonstrated that the gene responsible for enterotoxin A is located on the chromosome in two widely used strains of *S. aureus*. Strain FRI-100 had no extrachromosomal DNA and the other strain tested, S-6, still produced enterotoxin A after it had been cured of its single plasmid. The chromosomal locus for SEA was confirmed in 24 of 29 toxin-producing strains (Pattee and Glatz, 1980; Mallonee et al., 1982). The gene was mapped between the purine (*pur*) and isoleucine-valine (*ilv*) markers. However, the enterotoxin A gene could not be demonstrated in any of the analyzable linkage groups in the other five strains, and in at least four of these, plasmids were not demonstrable. Betley et al. (1984), employing hybridization analysis with a cloned SEA gene, detected at least two chromosomal locations for a gene-containing element. They believe the element represents a heterologous insertion that seems to be a "hotspot for structural rearrangements" and describe six different classes with respect to presence, type, and location: positive strains with a single intact copy located between the *pur* and *ilv* markers; positive strains with a single copy at another location; positive strains with an incomplete copy at the *pur-ilv* location and a functional copy elsewhere; positive strains with a rearranged genetic element unlinked to *pur-ilv*; negative strains with no homology; and negative strains with an incomplete copy at the *pur-ilv* location.

In an early study (Casman, 1965), one enterotoxin A-positive strain of *S. aureus*, PS42-D, was found to contain a prophage. Betley and Mekalanos (1985) have now confirmed that a phage derived from this strain could convert a nontoxigenic strain to a positive strain and find that the enterotoxin gene is associated with a phage in all positive strains examined. Hybridization analysis of the DNA from the phage from PS42-D and its bacterial host indicated that the phage integrated into the bacterial chromosome by circularization and reciprocal crossover. Some enterotoxin A genes were associated with either defective phage or phage with altered host range, and plaque-forming units could not be isolated from at least one enterotoxin-positive strain.

E. Genetic Regulation in Other Serotypes

A preliminary report by Betley and Bergdoll (1981) indicated that genes controlling production of enterotoxin C were associated with chromosomal DNA. Five strains (SEC-positive) were examined for plasmid DNA. Two cadmium-sensitive strains lacked detectable plasmids, while three cadmium-resistant strains each contained a single plasmid (each migrated differently, indicating that they were not the same species). After curing with

acriflavin, no plasmid DNA was detectable, but yields of enterotoxin C were not affected.

Recent information indicates that type D toxin may be plasmid-mediated (J. Iandolo, personal communication), but no information is available regarding genetic control of other serotypes of enterotoxins. Low yields and relatively insensitive assays make this work more difficult.

VII. MODE OF ACTION

Although numerous descriptive studies of the physiological effects of staphylococcal enterotoxins have been published (Elsberry et al., 1969; Hodoval et al., 1968; see Bergdoll, 1970), the molecular events mediating these effects remain unknown. The emetic response may result from a stimulation of nerve centers in the gut, transmitted to the vomiting center via the vagus and sympathetic nerves (Sugiyama and Hayama, 1965). The basis for the diarrheal response is even more nebulous: while the enterotoxins of *Vibrio cholerae* and *Escherichia coli* cause diarrhea by stimulating the adenylate cyclase of target cells, staphylococcal enterotoxins show no such stimulation (DeRubertis et al., 1974).

The interaction of SEB with a variety of cell culture lines has been the subject of several studies (Milone, 1962; Hallander and Bengtsson, 1967; Grigorova and Danon, 1968), but the results have been contradictory and, when positive, not very convincing. A cytotoxic effect of SEB on cultured human embryonic intestinal cells that could be completely neutralized by specific antitoxin was reported by Schaeffer et al. (1966). However, Schaeffer later found (1970) that the effect was reversible and depended critically on the serum with which the culture medium was supplemented. The effect of the enterotoxin was fully manifested only with human serum; calf serum-grown cells were resistant, and horse serum-grown cells were intermediate in reactivity. Not all human sera were equally effective, and the efficacy of positive batches diminished upon refrigerator storage. Finally, it has been pointed out that the cytopathic effect in cell culture may not be related to the in vivo enterotoxemia because of the very high levels required to bring it about (Gabliks, 1972).

In addition to in vivo gastrointestinal effect, staphylococcal enterotoxins A, B, and C_1 are potent mitogens (Peavy et al., 1970; Warren et al., 1975a), and Greaves et al. (1974) demonstrated that the effect is predominantly on T-cells. The enterotoxins (SEA has been employed most frequently) also stimulate the production of interferon from human and murine lymphocytes (Georgiades and Johnson, 1981a, 1981b). A specific receptor for SEA has been reported on murine spleen cells (Buxser et al., 1981). These authors also presented evidence suggesting that the different staphylococcal enterotoxins share a common receptor.

A recent report by Scheuber et al. (1985) opens a new and potentially important approach for investigating the site of toxic action. They observed that enterotoxin B, when administered intradermally to unsensitized monkeys, induced an immediate-type skin reaction in cutaneous mast cells. A nontoxic derivative with all its histidine residues blocked by carboxymethylation (see Sec. IV.B) completely abrogated the skin-sensitizing activity. It could compete with SEB for binding sites on the target cell surface.

The ability of SEB to elicit the release of vasoactive amines from mast cells was examined pharmacologically. A serotonin antagonist was ineffective, but an H1 competitive antagonist of histamine substantially inhibited the skin reaction, and cimetidine, a selective blocker of the H2 receptor, completely prevented the response. The skin reaction was also totally abrogated by diltriazem, a calcium channel blocker. Strikingly, it was observed that cimetidine and diltriazem completely prevented emesis and diarrhea upon gastric challenge with SEB.

These findings draw one's attention again to the highly conserved sequence in the enterotoxins starting at the end of the disulfide loop and containing the only conserved histidine residue in SEA, SEB, and SEC_1. A hydrophobic combining site with a positively charged activation-signal initiator is very attractive. This could not be the entire story of enterotoxin activity, because the pyrogenic exotoxin of *S. pyogenes* has essentially the same amino acid sequence. While there are some shared biological responses between this toxin and the enterotoxins, their primary effects are quite different.

VIII. IMMUNOLOGY

A. Cross-Reactivity

The original classification of the enterotoxins of *S. aureus* made on the basis of serologic individuality has proved to be simplistic and inadequate. Thus, the toxins produced by strains 137 and 361 were initially both called SEC, but careful serology demonstrated their nonidentity (Bergdoll et al., 1973). Prolonged interaction in Ouchterlony immunodiffusion gave rise to a spur when SEC_1 and SEC_2 in adjacent wells were reacted with antiserum to either toxin. Enterotoxin C_3 gives similar serological interactions with C_1 and C_2 (Reiser et al., 1984).

Common precipitating antibodies were found between SEA and SEE in antisera to both (Bergdoll et al., 1971). However, enterotoxins A and E each have unique immunogenic determinants not possessed by the other.

The lack of serologic cross-reaction does not, of course, preclude the presence of common antigenic determinants. This was first suggested by Gruber and Wright (1967) for enterotoxins A, B, and C_1. They reported that both SEA and SEC_1 were bound by antibody to SEB. Johnson et al. (1971) observed weak heterologous inhibition by SEA and SEC_1 of the SEB-anti-SEB system and by SEB and SEC_1 of the SEA-anti-SEA interaction. Spero et al. (1978) investigated the interaction of SEA, SEB, and SEC_1 with antisera to each enterotoxin by measurement of both antigen-binding capacity and the ability to bind competitively with homologous antigen. It was found that SEB and SEC_1 have a strong reciprocal reaction with each other's antibody. This is illustrated in Fig. 9 for anti-SEB and Fig. 10 for anti-SEC_1. All of the heterologous antigen combined, although about four times more antisera was required for each heterologous antigen over that required for the homologous interaction. The small apparent binding of SEA to anti-SEB is due to a low level of anti-SEA in the antiserum. The organism used for the production of SEB also elaborates small amounts of SEA and the protocol employed for the production of anti-SEB involves multiple injections over a long period of time. It was demonstrated, furthermore, that the traces of anti-SEA could be removed from the antiserum to enterotoxin B by affinity chromatography. This explains the capacity

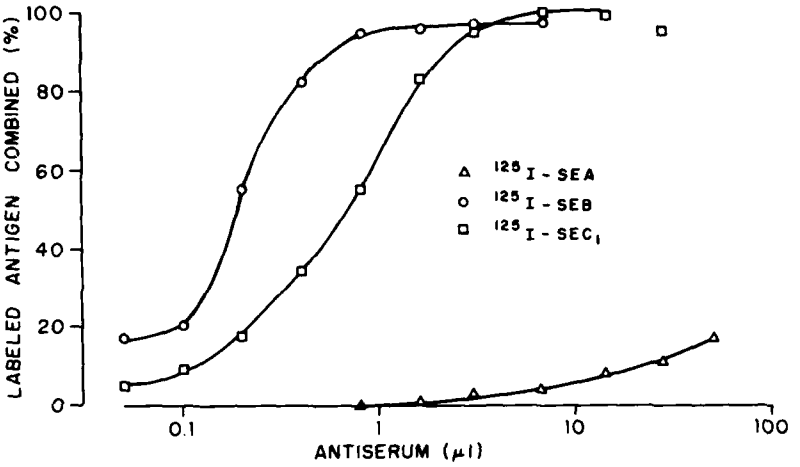

FIGURE 9 Binding of ^{125}I-labeled enterotoxins to rabbit antiserum to SEB (Spero et al., 1978).

of SEA for anti-SEB reported by Gruber and Wright (1967). In our laboratory, however, we were unable to find the heterologous SEA inhibition described by Johnson et al. (1971). It may be noteworthy that the enterotoxins and the antisera produced from them were not made in the same laboratory or by identical procedures.

The strong cross-reactions demonstrated for SEB and SEC_1 are reflected also in the well-defined polypeptides obtained by limited tryptic digestion of these enterotoxins (Spero and Morlock, 1978). The structure and nomenclature of these peptides as first obtained by Spero et al. (1976) and more precisely defined by Schmidt and Spero (1983) are described in

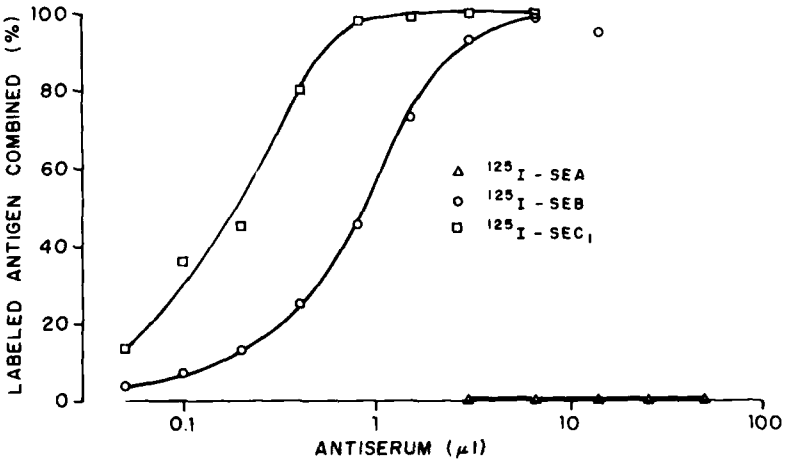

FIGURE 10 Binding of ^{125}I-labeled enterotoxins to rabbit antiserum to SEC_1 (Spero et al., 1978).

Sec. IV.B of this chapter. Measurement of the antigen-binding capacity of ^{125}I-labeled polypeptides (Spero and Morlock, 1979) established that there are two antigenic determinants on each enterotoxin capable of reacting with heterologous antibody: one on the amino-terminal peptide and one on the carboxyl-terminal peptide. The large C-terminal polypeptides bind efficiently to homologous antiserum, but about two orders of magnitude less efficiently to heterologous antibody. The N-terminal polypeptides show only weak homologous binding but nearly comparable heterologous binding. It was proposed that the determinant of the amino-terminal polypeptide is largely responsible for the strong reciprocal binding of the intact enterotoxins and that their low antigen-binding capacity is due to a random or structurally distorted conformation or structure. The N-terminal tryptic peptide from SEC_1 contains 57 residues. Compared to the corresponding residues of SEB, 42 are identical; indeed, there are only two differences in the first 30 residues.

Finally, it should be noted that in some antisera against types B and C_1, a cross-immunoprecipitation occurs (Lee et al., 1980). This observation demonstrates that at least three determinants may be possessed in common by these two serotypes.

B. Toxoids

The destruction of the toxicity of a protein antigen with retention of immunogenicity is the goal of the process called toxoiding. The most common reagent employed for this purpose is formaldehyde, and in 1966 Bergdoll reported the successful use of 0.7% formalin on SEB at pH 8.0 and 37°C for 3 weeks. In two very careful studies, Warren and co-workers (Warren et al., 1973, 1975b) approximated these conditions but varied the pH from 5.0 to 9.5 and extended the investigation to enterotoxins A and C_1. A composite of the results drawn from these papers is shown in Table 3.

The effect of the pH of inactivation on the preservation of the antigenic determinants was very similar for the three toxins. At pH 9.5, none remained; at pH 7.5, 15–20%; and at pH 5.0, 30–50% could be demonstrated by quantitative precipitin determination. In marked contrast were the differences observed upon their immunogenicity. SEA induced a poor response in the monkeys at all three pH values. SEB and SEC_1 were also very weak immunogens when formalinized at pH 9.5, but their preparations inactivated at pH 5.0 and 7.5 gave excellent responses.

Clearly we would not expect an immunological response to the pH 9.5 toxoids that were devoid of native determinants, but this parameter cannot explain the difference between the toxoids of SEA at pH 5.0 and 7.5 and those of SEB and SEC_1 prepared at the same pH values.

The data in the table demonstrate that a critical factor in the efficacy of enterotoxoids is the extent to which the proteins are polymerized. Thus the ineffective SEA toxoids consisted largely of small polymers (excluded by Sephadex G-100, but included by Sepharose 2B). The more highly polymerized pH 7.5 preparation of SEC, provoked an almost equally strong immune response as the pH 5.0 SEC_1 toxoid despite its much lower retention of antigenic determinants. The role of particle size was even more marked with SEB, where the extensively polymerized pH 7.5 toxoid was a significantly more potent immunogen than the less polymerized but much more antigenically active pH 5.0.

TABLE 3 Relation Between the Extent of Polymerization and Residual Antigenic Determinants and the Immunogenicity of Formalinized Staphylococcal Enterotoxins

Enterotoxin	*pH of formalinization*	Residual antigenic determinants (%)	Reciprocal titer (geometric mean)	Extent of polymerization
SEA	5.0	30	20	Small polymers
	7.5	20	20	Small polymers
	9.5	0	0	Monomer
SEB	5.0	50	∿160	30% large polymers
	7.5	15	∿640	90% large polymers
	9.5	0	0	Monomer
SEC_1	5.0	40	640	31% large polymers
	7.5	15	508	91% large polymers
	9.5	0	20	Monomer

This correlation of the degree of polymerization with immunogenicity has also been observed with other soluble protein antigens. However, it is not universally true. Thus, Relyveld (1978) found that in glutaraldehyde-inactivated diphtheria toxin, the dimer was more effective than either the monomer or a polymeric fraction.

IX. CONCLUSION

Over the last 15 years, significant accomplishments have been made in several aspects of staphylococcal enterotoxin research; for example, all of the known serotypes have been purified, SEC_1 has been chemically sequenced, the genes of both SEA and SEB have been cloned and their DNA sequenced, and the roles of plasmids and phage have been developed. However, major gaps in our knowledge remain. Foremost among these are the mode of action, the nature of the active site, and the three-dimensional structure of the molecules. Establishing the amino acid sequence of type E should not pose a great problem and, with the soon-to-be-published SEA sequence, a definition of the conserved residues in four serotypes will be of enormous value. The crystals of SEB recently obtained provide reason to hope for a successful x-ray diffraction study. The most urgent need is for a cell system with which the enterotoxins bind and/or induce a well-defined biochemical effect. It is to be hoped that the effect on cutaneous mast cells will provide a step in this direction.

X. RECENT DEVELOPMENTS

Since the completion of this review, the amino acid sequence of enterotoxin A, obtained by chemical means, has been published (Huang et al., 1987). A comparison with the sequences of SEB and SEC_1 suggests that neither the carboxy terminal nor the amino terminal regions play a role in the expression of emetic activity. Where 51 of the first 75 residues in SEB and SEC_1 are homologous, only 12 residues of SEA are identical (although the alignment of sequences is not unambiguous). Similarly, where 61 of the last 75 residues of SEB and SEC_1 are homologous, only 16 residues of SEA are identical.

The disulfide loop in SEA is strikingly smaller than in SEB or SEC_1: 9 residues in SEA, 19 in SEB, and 16 in SEC_1. This finding reinforces our conclusion that the loop is not involved in emetic activity.

On the other hand, the significance of the first grouping in our figure 5 is enhanced by the occurrence of the neighboring homologous lys-tyr-lys segment at residues 79–81 of SEA. Additionally, the new information on the sequence of SEA places a tyrosine at position 85 in our figure 5 (residue 88 in SEA). This eliminates the gap at position 91 in the top line and improves the homology between the residues.

A segment of potential importance is disclosed by sequence comparisons in the region near the second half-cystine, from residues 103 to 117 (SEA numbering). Herein 10 of the 15 residues are identical in all three serotypes, including a unique segment of five in succession. Finally, the area from 143 to 160, where 12 of 18 residues are homologous, might also be significant.

XI. REFERENCES

Avena, R. M. and Bergdoll, M. S. (1967). Purification and some physicochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. *Biochemistry 6*:1474–1480.

Baird-Parker, A. C. and Joseph, R. L. (1964). Fractionation of staphylococcal enterotoxin B. *Nature 202*:570–571.

Berdal, B. P., Olsvik, O. and Omland, T. (1962). A sandwich ELISA method for detection of *Staphylococcus aureus* enterotoxins. *Acta Pathol. Microbiol. Scand. B 89*:411–415.

Bergdoll. M. S. (1966). Immunization of rhesus monkeys with enterotoxoid B. *J. Infect. Dis. 116*:191–196.

Bergdoll, M. S. (1970). Enterotoxins. In *Microbial Toxins, III, Bacterial Protein Toxins*, T. C. Montie, S. Kadis, and S. J. Ajl (Eds.). Academic Press, New York, pp. 265–326.

Bergdoll, M. S., Borja, C. R., and Avena, R. M. (1965a). Identification of a new enterotoxin as enterotoxin C. *J. Bacteriol. 90*:1481–1485.

Bergdoll, M. S., Borja, C. R., Robbins, R., and Weiss, K. F. (1971). Identification of enterotoxin E. *Infect. Immun. 4*:593–595.

Bergdoll, M. S., Chu, F. S., Huang, I.-Y., Rowe, C., and Shih, T. (1965b). Staphylococcal enterotoxin B. III. The physicochemical properties and N- and C-terminal amino acid sequences. *Arch. Biochem. Biophys. 112*:104–110.

Bergdoll, M. S., Crass, B. A., Reiser, R. F., Robbins, R. N., and Davis, J. P. (1981). A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock syndrome isolates. *Lancet 1*:1017–1021.

Bergdoll, M. S., Robbins, R. N., Weiss, K., Borja, C. R., Huang, I.-Y., and Chu, F. S. (1973). The staphylococcal enterotoxins: Similarities. In *Contributions to Microbiology and Immunology, Vol. 1, Staphylococci and Staphylococcal Infections*, J. Jeljaszewicz (Ed.). S. Karger, Basel, pp. 390–396.

Bergdoll, M. S., Surgalla, M. J., and Dack, G. M. (1959). Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin-neutralizing property. *J. Immunol. 83*:334–338.

Betley, M. J., and Bergdoll, M. S. (1981). Staphylococcal enterotoxin type C genes not associated with extrachromosomal DNA. Abstracts, Annual Meeting ASM, Dallas, TX, p. 49.

Betley, M. J., and Mekalanos, J. J. (1985). Staphylococcal enterotoxin A is encoded by phage. *Science 229*:185–187.

Betley, M. J., Lofdahl, S., Kreiswirth, B. N., Bergdoll, M. S., and Novick, R. P. (1984). Staphylococcal enterotoxin A gene is associated with a variable genetic element. *Proc. Natl. Acad. Sci. USA 81*:5179–5183.

Blobel, G., and Dobberstein, B. (1975). Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains by membrane-bound ribosomes of murine myeloma. *J. Cell Biol. 67*:835–851.

Borja, C. R. (1969). Staphylococcal enterotoxin C. I. Phenolic hydroxyl ionization. *Biochemistry 8*:71–75.

Borja, C. R. and Bergdoll, M. S. (1967). Purification and partial characterization of enterotoxin C produced by *Staphylococcus aureus* strain 137. *Biochemistry 6*:1467–1473.

Borja, C. R., Fanning, E., Huang, I.-Y., and Bergdoll, M. S. (1972). Purification and some physicochemical properties of staphylococcal enterotoxin E. *J. Biol. Chem. 247*:2456–2463.

Buxser, S., Bonventre, P. F., and Archer, D. L. (1981). Specific receptor binding of staphylococcal enterotoxins by murine splenic lymphocytes. *Infect. Immun. 33*:827–833.

Casman, E. P. (1960). Further serological studies of staphylococcal enterotoxin. *J. Bacteriol. 79*:849–856.

Casman, E. P., Bennett, R. W., Dorsey, A. E., and Issa, J. A. (1967). Identification of a fourth staphylococcal enterotoxin, enterotoxin D. *J. Bacteriol. 94*:1875–1882.

Casman, E. P., Bergdoll, M. S., and Robinson, J. (1963). Designation of staphylococcal enterotoxins. *J. Bacteriol. 85*:715–716.

Center for Disease Control (1981). Food-borne disease outbreaks. *In Annual Summary*, Atlanta, GA.

Chang, H. C. and Bergdoll, M. S. (1979). Purification and some physicochemical properties of staphylococcal enterotoxin D. *Biochemistry 10*: 1937–1942.

Chang, P. C. and Dickie, N. (1971). Fractionation of staphylococcal enterotoxin B by isoelectric focusing. *Biochim. Biophys. Acta 236*:367–375.

Christianson, K. K., Tweten, R. K., and Iandolo, J. J. (1985). Transport and processing of staphylococcal enterotoxin A. *Appl. Environ. Microbiol. 50*:696–697.

Chu, F. S. (1968). Hydrogen ion equilibria of staphylococcal enterotoxin B. *J. Biol. Chem. 243*:4341–4349.

Chu, F. S. and Bergdoll, M. S. (1969). Studies on the chemical modification of staphylococcal enterotoxin B. I. Alkylation and oxidation of methionine residues. *Biochim. Biophys. Acta 194*:279–286.

Chu, F. S. and Crary, E. (1969). Studies on the chemical modification of staphylococcal enterotoxin B. *Biochim. Biophys. Acta 194*:287–292.

Chu, R. S., Crary, E., and Bergdoll, M. S. (1969). Chemical modification of amino groups in staphylococcal enterotoxin B. *Biochemistry 8*:2890–2896.

Chu, F. S., Thadhani, K., Schantz, E. J., and Bergdoll, M. S. (1966). Purification and characterization of staphylococcal enterotoxin A. *Biochemistry* 5:3281–3289.

Collins, W. S., Metzger, J. F., and Johnson, A. D. (1972). A rapid solid phase radioimmunoassay for staphylococcal B enterotoxin. *J. Immunol. 108*:852–856.

Dalidowicz, J. D., Silverman, S. J., Schantz, E. J., Stefanye, D., and Spero, L. (1966). Chemical and biological properties of reduced and alkylated staphylococcal enterotoxin B. *Biochemistry* 5:2375–2381.

DeRubertis, F. R., Zenser, T. V., Adler, W. H., and Hudson, T. (1974). Role of cyclic adenosine 3',5'-monophosphate in lymphocyte mitogenesis. *J. Immunol. 113*:151–161.

Dorbusch, K., Hallander, H. O., and Lofquist, F. (1969). Extra chromosomal control of methicillin resistance and toxin production in *Staphylococcus aureus*. *J. Bacteriol. 98*:351–358.

Dyer, D. W. and Iandolo, J. J. (1981). Plasmid-chromosomal transition of genes important in staphylococcal B expression. *Infect. Immun. 33*: 450–458.

Dyer, D. W. and Iandolo, J. J. (1982). The distribution of enterotoxin B related plamids among species of staphylococcus. Abstracts. Annual Meeting ASM, Atlanta, GA, p. 54.

Elsberry, D. D., Rhoda, D. A., and Beisel, W. R. (1969). Hemodynamics of staphylococcal B enterotoxemia and other types of shock in monkeys. *J. Appl. Physiol. 27*:164–169.

Fey, H., Pfister, H., and Ruegg, O. (1984). Comparative evaluation of different enzyme-linked immunosorbent assay systems for the detection of staphylococcal enterotoxins A, B, C, and D. *J. Clin. Microbiol. 19*:34–38.

Friedman, M. E. and Howard, M. B. (1971). Induction of mutants of *Staphylococcus aureus* 100 with increased ability to produce enterotoxin A. *J. Bacteriol. 106*:289–291.

Gabliks, J. (1972). Protective action of 2,2-bis (parachlorophenyl)-1,1,1-trichloroethane against intoxication of cells by staphylococcal enterotoxin B. *Infect. Immun. 6*:364–369.

Georgiades, J. A. and Johnson, H. M. (1981a). Partial purification of human immune interferon. *Methods Enzymol. 78*:536–539.

Georgiades, J. A. and Johnson, H. M. (1981b). Partial purification and characterization of mouse immune interferon. *Methods Enzymol. 78*: 545–552.

Greaves, M., Janossy, G., and Doenhoff, M. (1974). Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens. *J. Exp. Med. 140*:1–17.

Greenwood, F. C and Hunter, W. M. (1963). The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J. 89*:114–123.

Grigorova, M. and Danon, S. (1968). The effect of staphylococcal enterotoxin on tissue cultures. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. 206*:478–481.

Gruber, J. and Wright, G. G. (1967). Iodine-131 labeling of purified microbial antigens by microdiffusion. *Proc. Soc. Exp. Biol. Med. 126*: 282–284.

Hallander, H. O. and Bengtsson, S. (1967). Studies on the cell toxicity and species specificity of purified staphylococcal toxins. *Acta Pathol. Microbiol. Scand. 70*:107–119.

Hodoval, L. F., Morris, E. L., Crawley, G. J., and Beisel, W. R. (1968). Pathogenesis of lethal shock after intravenous staphylococcal enterotoxin B in monkeys. *Appl. Microbiol. 16*:187–192.

Huang, I.-Y. and Bergdoll. M. S. (1970). The primary structure of staphylococcal enterotoxin B. III. The cyanogen bromide peptides of reduced and aminoethylated enterotoxin B, and the complete amino acid sequence. *J. Biol. Chem. 245*:3518–3525.

Huang, I. Y., Hughes, J. L., Bergdoll, M. S. and Schantz, E. J., (1987). Complete amino acid sequence of staphylococcal enterotoxin A. *J. Biol. Chem. 262*:7006–7013.

Huang, I.-Y., Schantz, E. J., and Bergdoll, M. S. (1975). The amino acid sequence of the staphylococcal enterotoxins. *Jpn. J. Med. Sci. Biol. 28*:73–75.

Iandolo, J. J. and Dyer, D. W. (1981). The staphylococcal enterotoxins-a genetic overview. *J. Food Safety 3*:249–264.

Johnson, H. M., Bucovic, J. A., Kauffman, P. E., and Peeker, J. T. (1971). Staphylococcal enterotoxin B: solid phase radioimmunoassay. *Appl. Microbiol. 22*:837–841.

Johnson, L. P., L'Italien, J. J. and Schlievert, P. M. (1986). Streptococcal pyrogenic exotoxin type A (scarlet fever toxin) is related to *Staphylococcus aureus* enterotoxin B. *Mol. Gen. Genet. 203*:354–356.

Jones, C. L. and Khan, S. A. (1986). Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. *J. Bacteriol. 166*:29–33.

Jonsson, S. and Kronvall, G. (1974). The use of protein-A containing *Staphylococcus aureus* as a solid phase anti-IgG reagent in radioimmunoassays as exemplified in the quantitation of α-feto protein in normal human serum. *Eur. J. Immunol. 4*:29–33.

Khan, S. A. and Novick, R. P. (1982). Structural analysis of plasmid pSN2 in *Staphylococcus aureus*: No involvement in enterotoxin B production. *J. Bacteriol. 149*:642–649.

Kuntz, I. D. (1972). Protein folding. *J. Am. Chem. Soc. 94*:4009–4012.

Langford, M. P., Stanton, G. J., and Johnson, H. M. (1978). Biological effects of staphylococcal enterotoxin A on human peripheral lymphocytes. *Infect. Immun. 22*:62–68.

Lee, A. C.-M., Robbins, R. N., Reiser, R. F., and Bergdoll, M. S. (1980). Isolation of specific and common antibodies to staphylococcal enterotoxins B, C_1, and C_2. *Infect. Immun. 27*:431–434.

Mallonee, D. H., Glatz, B. A., and Pattee, P. A. (1982). Chromosomal mapping of a gene affecting enterotoxin A production in *Staphylococcus aureus*. *Appl. Environ. Microbiol. 43*:397–402.

Metzger, J. F., Johnson, A. D., and Collins, W. S. (1972). Fractionation and purification of *Staphylococcus aureus* enterotoxin B by electrofocusing. *Biochim. Biophys. Acta 257*:183–186.

Metzger, J. F. Johnson, A. D., and Spero, L. (1975). Intrinsic and chemically produced microheterogeneity of *Staphylococcus aureus* enterotoxin type C. *Infect. Immun. 12*:93–97.

Middlebrook, J. L., Spero, L., and Argos, P. (1980). The secondary structure of staphylococcal enterotoxins A, B, and C. *Biochim. Biophys. Acta 621*:233–240.

Milone, N. A. (1962). Studies on the use of tissue culture for the bioassay of staphylococcal enterotoxin. *J. Food Sci. 27*:501–507.

Muñoz, P. A., Warren, J. R., and Noelken, M. D. (1976). β-Structure of aqueous staphylococcal enterotoxin B by spectropolarimetry and sequence-based conformational predictions. *Biochemistry 15*:4666–4671.

Niskanen, A. and Lindroth, S. (1976). Comparison of different purification procedures for extraction of SEA from foods. *Appl. Environ. Microbiol. 32*:455–464.

Novick, R. P., Sanchez-Rivas, C., Gruss, A., and Edelman, I. (1980). Involvement of the cell envelope in plasmid maintenance: Plasmid curing during the regeneration of protoplasts. *Plasmid 3*:348–358.

Pattee, P. A. and Glatz, B. A. (1980). Identification of a chromosomal determinant of enterotoxin A products in *Staphylococcus aureus*. *Appl. Environ. Microbiol. 39*:186–193.

Peavy, D. L., Adler, W. H., and Smith, R. T. (1970). The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. *J. Immunol. 105*:1453–1458.

Ranelli, D. M., Jones, C. L., Johns, M. B., Mussey, G. J., and Khan, S. A. (1985). Molecular cloning of staphylococcal enterotoxin B gene in *Escherichia coli* and *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA 82*:5850–5854.

Reiser, R. F., Robbins, R. N., Khoe, G. P., and Bergdoll, M. S. (1983). Purification and some physicochemical properties of toxic-shock toxin. *Biochemistry 22*:3907–3912.

Reiser, R. F., Robbins, R. N., Noleto, A. L., Khoe, G. P., and Bergdoll, M. S. (1984). Identification, purification, and some physicochemical properties of staphylococcal enterotoxin C_3. *Infect. Immun.* 45:625–630.

Relyveld, E. H. (1978). Detoxification of microbial toxins with glutaraldehyde and their use in the preparation of vaccines. In *Toxins: Animal, Plant, and Microbial*, Philip Rosenberg (Ed). Pergamon Press, Elmsford, NY, pp. 1049–1065.

Richardson, J. S. (1981). The anatomy and taxonomy of protein structure. *Adv. Protein Chem.* 34:168–340.

Robern, H., Dighton, M., Yano, Y., and Dickie, N. (1975). Double-antibody radioimmunoassay for staphylococcal enterotoxin C_2. *Appl. Microbiol.* 30:525–529.

Schaeffer, W. I. (1970). Interaction of staphylococcal enterotoxin B with cell cultures. I. Effect of serum and testing procedures. *Infect. Immun.* 1:455–458.

Schaeffer, W. I., Gabliks, J., and Calitis, R. (1966). Interaction of staphylococcal enterotoxin B with cell cultures of human embryonic tissue. *J. Bacteriol.* 91:21–26.

Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A., and Bergdoll, M. S. (1965). Purification of staphylococcal enterotoxin B. *Biochemistry* 4:1011–1016.

Schantz, E. J., Roessler, W. G., Woodburn, M. J., Lynch, J. M., Jacoby, H. M., Silverman, S. J., Gorman, J. C., and Spero, L. (1972). Purification and some chemical and physical properties of staphylococcal enterotoxin A. *Biochemistry* 11:360–366.

Scheuber, P. H., Golecki, J. R. Kickhofen, F., Scheel, D., Beck, G., and Hammer, D. K. (1985). Skin reactivity of unsensitized monkeys upon challenge with staphylococcal enterotoxin B: A new approach for investigating the site of toxic action. *Infect. Immun.* 50:869–876.

Schmidt, J. J. and Spero, L. (1983). The complete amino acid sequence of enterotoxin C_1. *J. Biol. Chem.* 258:6300–6306.

Shafer, W. M. and Iandolo, J. J. (1978a). Chromosomal locus for staphylococcal enterotoxin B. *Infect. Immun.* 20:273–278.

Shafer, W. M. and Iandolo, J. J. (1978b). Staphylococcal enterotoxin A: A chromosomal gene product. *Appl. Environ. Microbiol.* 36:389–391.

Shafer, W. M. and Iandolo, J. J. (1979). Genetics of enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. *Infect. Immun.* 25:920–911.

Shalita, A., Hertman, I., and Sarid, S. (1977). Isolation and characterization of plasmid involved with enterotoxin B production in *Staphylococcus aureus*. *J. Bacteriol.* 129:317–325.

Spero, L. (1981). Analysis of the near-ultraviolet circular dichroic spectra of staphylococcal entertoxins A, B, and C. *Biochim. Biophys. Acta* 671:193–201.

Spero, L. and Metzger, J. F. (1981). Staphylococcal enterotoxin A (SEA). *Methods Enzymol.* 78:331–336.

Spero. L. and Morlock B. A. (1978). Biological activity of the peptides of staphylococcal enterotoxin C formed by limited tryptic hydrolysis. *J. Biol. Chem.* 253:8787–8791.

Spero, L. and Morlock, B. A. (1979). Cross-reactions between tryptic polypeptides of staphylococcal enterotoxins B and C. *J. Immunol.* 122: 1285–1289.

Spero, L., Griffin, B. Y., Middlebrook, J. L., and Metzger, J. F. (1976). Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin C. *J. Biol. Chem. 251*:5580–5588.

Spero, L., Jacoby, H. M., Dalidowicz, J. E., and Silverman, S. J. (1971). Guanidination and nitroguanidination of staphylococcal enterotoxin B. *Biochim. Biophys. Acta 251*:345–354.

Spero, L., Leatherman, D. L., and Adler, W. H. (1975a). Mitogenicity of formalinized toxoids of staphylococcal enterotoxin B. *Infect. Immun. 12*:1018–1020.

Spero, L., Metzger, J. F., Warren, J. R., and Griffin, B. Y. (1975b). Biological activity and complementation of the two peptides of staphylococcal enterotoxin B formed by limited tryptic hydrolysis. *J. Biol. Chem. 250*:5026–5032.

Spero, L., Morlock, B. A., and Metzger, J. F. (1978). On the cross-reactivity of staphylococcal enterotoxins A, B, and C. *J. Immunol. 120*: 86–89.

Spero, L, Warren, J. R., and Metzger, J. F. (1973). Effect of a single peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin B. *J. Biol. Chem. 248*:7289–7294.

Spero, L., Warren, J. R., and Metzger, J. F. (1974). Microheterogeneity of staphylococcal enterotoxin B. *Biochim. Biophys. Acta 336*:79–85.

Stelma, G. N. and Bergdoll, M. S. (1982). Inactivation of staphylococcal enterotoxin A by chemical modification. *Biochem. Biophys. Res. Commun. 105*:121–126.

Sugiyama, H. and Hayama, T. (1965). Abdominal viscera as site of emetic action for staphylococcal enterotoxin in the monkey. *J. Infect. Dis. 115*:330–336.

Tweten, R. K. and Iandolo, J. J. (1981). Purification and partial characterization of a putative precursor to staphylococcal enterotoxin B. *Infect. Immun. 34*:900–907.

Tweten, R. K. and Iandolo, J. J. (1983). Transport and processing of staphylococcal enterotoxin B. *J. Bacteriol. 153*:297–303.

Wagman, J., Edwards, R. C., and Schantz, E. J. (1965). Molecular size, homogeneity, and hydrodynamic properties of purified staphylococcal enterotoxin B. *Biochemsitry 4*:1017–1023.

Warren, J. R., Spero, L., and Metzger, J. F. (1973). Antigenicity of formaldehyde-inactivated staphylococcal enterotoxin B. *J. Immunol. 111*:885–892.

Warren, J. R., Spero, L., and Metzger, J. F. (1974). Stabilization of native structure by the closed disulfide loop of staphylococcal enterotoxin B. *Biochim. Biophys. Acta 359*:351–363.

Warren, J. R., Leatherman, D. L., and Metzger, J. F. (1975a). Evidence for cell-receptor activity in lymphocyte stimulation by staphylococcal enterotoxin. *J. Immunol. 115*:49–53.

Warren, J. R., Spero, L., Metzger, J. F., and McGann, V. G. (1975b). Immunogenicity of formaldehyde-inactivated enterotoxins A and C_1 of *Staphylococcus aureus*. *J. Infect. Dis. 131*:535–542.

Weeks, C. R. and Ferretti, J. J. (1986). Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T12. *Infect. Immun.* *52*:144–150.

Wofsy, L. and Singer, S. J. (1963). Effects of the amidination reaction on antibody activity and on the physical properties of some proteins. *Biochemistry* *2*:104–116.